Unusual Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase of Anoxic *Archaea*

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The predominant pool of organic matter on earth is derived from the biological reduction and assimilation of carbon dioxide gas, catalyzed primarily by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). By virtue of its capacity to use molecular oxygen as an alternative and competing gaseous substrate, the catalytic efficiency of RubisCO and the enzyme's ability to assimilate CO_2 may be severely limited, with consequent environmental and agricultural effects. Recent genomic sequencing projects, however, have identified putative RubisCO genes from anoxic *Archaea*. In the present study, these potential RubisCO sequences, from *Methanococcus jannaschii* and *Archaeoglobus fulgidus*, were analyzed in order to ascertain whether such sequences might encode functional proteins. We also report the isolation and properties of recombinant RubisCO using sequences obtained from the obligately anaerobic hyperthermophilic methanogen *M. jannaschii*. This is the first description of an archaeal RubisCO sequence; this study also represents the initial characterization of a RubisCO molecule that has evolved in the absence of molecular oxygen. The enzyme was shown to be a homodimer whose deduced sequence, along with other recently obtained archaeal RubisCO sequences, differs substantially from those of known RubisCO molecules. The recombinant *M. jannaschii* enzyme has a somewhat low, but reasonable k_{cat}, however, unlike previously isolated RubisCO molecules, this enzyme is very oxygen sensitive yet it is stable to hyperthermal temperatures and catalyzes the formation of the expected carboxylation product. Despite inhibition by oxygen, this unusual RubisCO still catalyzes a weak yet demonstrable oxygenase activity, with perhaps the lowest capacity for CO_2/O_2 discrimination ever encountered for any RubisCO.

Carbon dioxide (CO_2) is a greenhouse gas whose relative concentration is thought to be increasing in the earth's atmosphere (23). CO_2 is also the sole carbon source for the predominant life-forms on this planet, and its efficient incorporation into organic matter is directly related to the productivity of important ecosystems, including agriculturally significant plants (34). For most organisms, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) catalyzes the primary step of CO_2 fixation (6, 8, 25); despite the fact that it is the most abundant protein found on earth (3), RubisCO's catalytic efficiency is severely limited by the capacity to catalyze a competing O_2 fixation reaction. This leads to inefficient CO_2 fixation and low productivity. At this time, the molecular basis for CO_2/O_2 discrimination is not completely understood; however, the relative capacity of this enzyme to favor either carboxylation or oxygenation is not immutable but varies for different sources of RubisCO (9, 20). Recently, the complete genomes of the hyperthermophilic archaeons *Methanococcus jannaschii* and *Archaeoglobus fulgidus* were sequenced (2, 10). These organisms are representative of prokaryotic organisms (*Archaea*) that are considered to be distinct from eubacteria; they also possess many characteristics common to eukaryotes and are thus thought to be representative of a third kingdom of life (31). Since both methanogenic and sulfate-reducing archaea are anoxic organisms that fix CO_2 by acetyl coenzyme A and reductive tricarboxylic acid-like pathways (5, 22, 24, 32), it was most surprising to find that both genomes contain sequences that potentially encode the large subunit of RubisCO.

Indeed, *A. fulgidus* possesses two putative RubisCO genes. RubisCO is the key enzyme of the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway (6, 8, 25), a route that is quite distinct from the known CO_2 assimilatory pathways of these organisms. Despite the fact that the CBB pathway and RubisCO are undoubtedly responsible for the bulk of organic carbon on earth, the CBB route has never been unequivocally established to be significant in obligately anoxic prokaryotes. Since RubisCO also catalyzes a physiologically important oxygenase reaction, such that CO_2 and O_2 compete for the enzyme-bound enediolate of RuBP (6, 8, 25), it is perhaps understandable that obligately anoxic prokaryotes employ other CO_2 fixation reaction schemes. However, the unexpected potential opportunity to examine RubisCO from organisms that evolved in the complete absence of oxygen presumably provides an unprecedented opportunity to discern how the active site of diverse RubisCO enzymes may have evolved to function at different CO_2 and O_2 tensions. This study was directed at examining the potential of archaeal RubisCO sequences to encode for functional enzymes. The results of this study indicate that recombinant *M. jannaschii* RubisCO catalyzes a bonafide RubisCO reaction, albeit with many unusual properties, including a rather unique interaction with oxygen.

MATERIALS AND METHODS

Expression vector construction. An *M. jannaschii* RubisCO expression plasmid was constructed beginning with plasmid pAMJEV50, which was obtained from The Institute for Genomic Research, Rockville, Md. It contains the putative RubisCO large subunit gene, MJ1235, plus another hypothetical open reading frame, MJ1234. After being treated with *Ase*I, plasmid pAMJEV50 yielded a 1.4-kb fragment containing the *M. jannaschii* RubisCO gene plus a truncated fragment of MJ1234. The 5′ overhangs of the *Ase*I fragment were filled and subcloned into plasmid pK18 by using the *Sma*I site of the multicloning region.

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After sequencing to establish orientation, the resulting plasmid, pK18GW, was digested with *Bam*HI and *Kpn*I and ligated into the (His)₆-tagged vector pPROEXHTa (Life Technologies, Inc., Gaithersburg, Md.) to produce expression vector pGWa1.

Synthesis and purification of recombinant RubisCO. The recombinant *M. jannaschii* RubisCO was purified from crude extracts of *Escherichia coli* DH5α (pGWa1) grown anaerobically at 35°C in Terrific (glycerol)-fumarate growth medium containing (per liter of distilled water) 12 g of typtone, 24 g of yeast extract, 4 g of fumaric acid, and 0.5% (vol/vol) glycerol, buffered at pH 7.2 with 90 mM potassium phosphate buffer containing 100 μg of ampicillin/ml. Seed cultures of 100 ml were grown aerobically in shake flasks overnight and inoculated into a fermenter vessel containing 10 liters of medium which was sparged with either nitrogen or argon gas at a rate of 2 liters/h. When the culture reached an optical density at 590 nm (OD₅₉₀) of between 0.2 and 0.3, isopropyl β-D-galactopyranoside was added to a concentration of 0.1 mM. After the culture reached an OD₅₉₀ of between 0.4 and 0.6, the cells were harvested anaerobically by using a continuous flow centrifuge system flushed with nitrogen gas. The cell pellet was placed in a 250-ml centrifuge bottle in an anaerobic chamber and then stored at −70°C. At the desired time, the frozen cells were thawed and centrifuged anaerobically at 16,000 × *g* at 4°C for 10 min to remove leftover broth. The cell pellet was washed twice anaerobically with 250 ml of lysis buffer (50 mM Tris-HCl [pH 8.5] containing 10 mM β-mercaptoethanol and 0.8 M KC1). The washed cell pellet, in 50 ml of lysis buffer containing 1 mM phenylmethysulfonyl fluoride, was disrupted by using a pressurized French pressure cell (at 102,000 kPa) with the crude extract extruded into a 160-ml sealed serum bottle flushed with argon gas. The crude extract was placed in a 250-ml centrifuge bottle in the anaerobic hood, which was then sealed and centrifuged at 16,000 × *g* at 4°C for 10 min. The supernatant was decanted into a fresh 160-ml serum bottle in the anaerobic hood; the bottle was pressurized with argon to 68 kPa and then placed into an 85°C water bath for 15 min with gentle shaking. The heat-treated crude extract was transferred to 50-ml anaerobic centrifuge tubes and centrifuged at 30,000 × *g* for 30 min at 4°C. This supernatant was added to an argon-pressurized (68 kPa) 160-ml serum bottle and stored at 4°C or used (at room temperature) for the subsequent column chromatography step. Nickel chelate chromatography, following protocols provided with the ProEX HT Prokaryotic Expression System kit (Life Technologies, Inc.), was performed in the anaerobic hood. Fractions were collected in 2-ml sealed serum vials, and samples were assayed for activity by standard procedures. Recombinant *Rhodospirillum rubrum* RubisCO was purified by a previously established procedure (27) except that a Green-A agarose dye affinity column was substituted for the DEAE-cellulose column step (19). Polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) was performed by standard procedures (20), at 10 and 8% acrylamide, respectively. In some cases, nondenaturing gradient gels (from 4 to 20% acrylamide) were used.

Analysis and quantitation of reaction products. Radiometric ¹⁴CO₂ fixation and coupled 3-phosphoglyceric acid (3-PGA) assays were performed as previously described (26). The transition state analog 2-carboxyarabinitol 1,5-bisphosphate (CABP) and [1-³H]RuBP were synthesized by established procedures (12, 17). To quantitate the level of RubisCO reaction products, [1-³H]RuBP (80 μM) was incubated with the *M. jannaschii* enzyme (66 μg/ml) or the *R. rubrum* enzyme (2 μg/ml) in 0.5 ml of 80 mM HEPES-NaOH buffer, pH 7.2, containing 10 mM β-mercaptoethanol. The reactions were quenched with NaBH₄, and the mixtures were deproteinated and applied and eluted from a MonoQ anion exchange column (HR5/5, 5 by 50 mm; Pharmacia Biotech, Inc., Piscataway, N.J.), as previously described (7). Radioactivity was continuously monitored with a β-RAM detector (IN/US, Tampa, Fla.).

RESULTS AND DISCUSSION

Analysis of potential archaeal RubisCO sequences. As a first attempt to determine if the archaeal sequences encode active RubisCO, we carefully analyzed the *M. jannaschii* and *A. fulgidus* deduced amino acid sequences. Phylogenetic analysis (Fig. 1) of representatives of all known classes of RubisCO shows that the putative *M. jannaschii* and *A. fulgidus* proteins fall into a group that is quite distinct from previous groupings of known form I and form II enzymes (30). Indeed, the distinctness of the *M. jannaschii* and *A. fulgidus* deduced sequences are such that one might question whether they could possibly encode functional proteins. The putative proteins possessed characteristic motifs of both form I and form II RubisCO large subunits, the two structurally distinct types of catalytic polypeptide heretofore described (6, 8, 25) (Fig. 2). The *M. jannaschii* protein shows 33% amino acid identity to a typical form II subunit (that from the nonsulfur purple bacterium *R. rubrum*) and exhibits 41% identity to the closest representative form I RubisCO large subunit (that from the cyanobacterium *Synechococcus* sp.
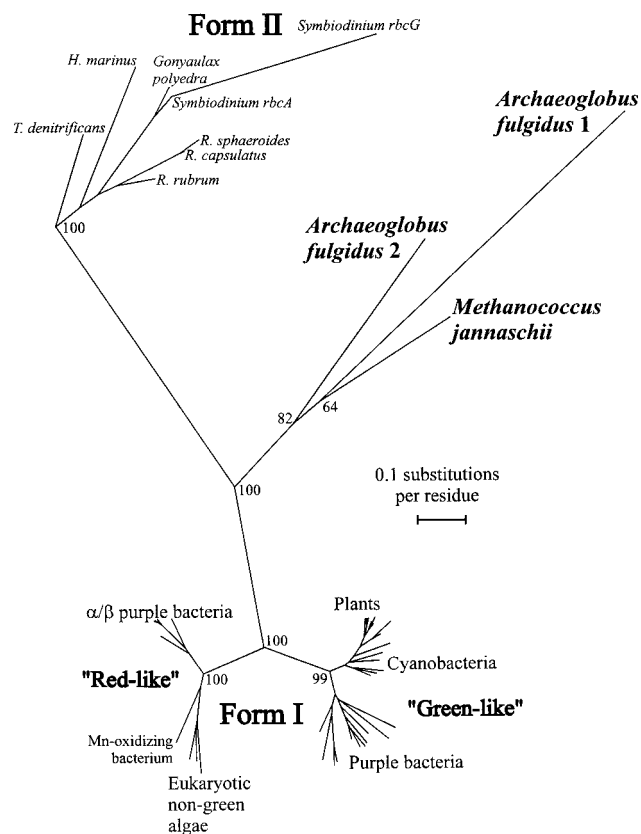

FIG. 1. Molecular phylogenetic tree derived from selected RubisCO large subunit amino acid sequences. Tree topology and evolutionary distance estimations were performed by the neighbor-joining method with Kimura distances (Phylip 3.5) (4). This tree is unrooted. Bootstrap values, calculated from 1,000 replicates, are indicated at major nodes of the tree and are expressed as percentages.

strain PCC 6301); the *R. rubrum* and *Synechococcus* sequences are themselves 33% identical. The crystal structures of both these bacterial RubisCO have been solved (14, 15). Interestingly, the *A. fulgidus* 1 and *A. fulgidus* 2 deduced sequences show only 41 and 45% identity, respectively, to the putative *M. jannaschii* RubisCO. However, comparisons of the deduced amino acid sequences of the *M. jannaschii* and *A. fulgidus* RubisCOs to the *Synechococcus* form I and *R. rubrum* form II enzymes (Fig. 2) indicated that almost all the critical active-site residues were present. For example, of 14 active-site residues (Fig. 3) determined to be within 3.3 Å of the bound transition state analog CABP (15) in the *Synechococcus* enzyme, 13 residues are identical in the *M. jannaschii* and *A. fulgidus* sequences. The only variant residues are at position 193 of the alignment (Fig. 2). It should be noted that the phenylalanine in the consensus pattern G-x-D-F-x-K-x-D-E, found in all functional RubisCOs so far identified, is replaced by a leucine in the *M. jannaschii* sequence and by isoleucine or tyrosine in the *A. fulgidus* proteins. While the lysine becomes carbamylated during "activation" of the enzyme and the second aspartate is a magnesium ligand (1), to our knowledge the phenylalanine of this motif has not been shown to be directly required for catalysis. Although the deduced sequences of the putative archaeal RubisCO proteins are more similar to that of the form I enzyme, no small subunit sequence was detected in either genome. Consequently, we have found that the putative archaeal proteins contain either

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Synechococcus PCC6301  MPKTQSAAGYKAGVKDYKLTYYTPDYTPKDTD----LLAAFPVSPQPGVPADEAGAAIAA   60
M. jannaschii                     ------------MGEGYTMDYINLNYRPNEGD----LLSCMVIK---GENLEKLANEIAG
A. fulgidus 1             -----MQLGVRLRFQKFEYPEANPEALPEGIDPEEYIIGTYYMSFPKGMNPFEITQVLAL
A. fulgidus 2             -----------MAEFEIYREYVDKSYEPQKDD----IVAVFRITPAEGFTIEDAAGAVAA
R. rubrum                 ---------MDQSSRYVNLALKEEDLIAGGEH----VLCAYIMKPKAGYGYVATAAHFAA
                                            .   .   .   ::     :.   *         .*


                           A
Synechococcus PCC6301  ESSTGTWTTVWTDLLTD-MDRYKGKCYHIEPVQG------EENS---YFAFIAYPLDLFE 120
M. jannaschii          ESSIGTWTKVQTMKS-DIYEKLRPKVYEIKEIG-------EENGYKVGLIKIAYPLYDFE
A. fulgidus 1          EQSTGTWLPVPGETP-EVRRKHVAKVVGVYEIPDYEIMVPQEVDWRNFIVQIAFPWRNIG
A. fulgidus 2          ESSTGTWTSLHPWYDEERVKGLSAKAYDFVDLG-------DGS----SIVRIAYPSELFE
R. rubrum              ESSTG--TNVEVCTTDDFTRGVDALVYEVDEAR-------------ELTKIAYPVALFH
                       *.* *    :       :         .              :  **:*    :


                                                                    A                   S    S  S
Synechococcus PCC6301  E------GSVTNILTSIVGNVFGFKAIRSLRLEDIRFPVALVKTFQGPPHGIQVERDLLN 180
M. jannaschii          I------NNMPGVLAGIAGNIFGMKIAKGLRILDFRFPAEFVKAYKGPRFGIEGVRETLK
A. fulgidus 1          -------SKLSMLFSTVVGNIS---MAPKLKLLDLRFPKEFVKGFKGPKFGIEGVRDVLG
A. fulgidus 2          P------HNMPGLLASIAGNVFGMKRVKGLRLEDLQLPKSFLKDFKGPSKGKEGVKKIFG
R. rubrum              RNITDGKAMIASFLTLTMGNNQGMGDVEYAKMHDFYVPEAYRALFDGPSVNISALWKVLG
                              :. .::     **         :: *:  .*       :.**  .  . .   . :


                       SS       S      A   A  A                S   S  GXDFXKXDE
Synechococcus PCC6301  KY---GRPMLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENINSQPFQRWRDRFLF 240
M. jannaschii          IK---ERPLLGTIVKPKVGLKTEEHAKVAYEAWVGGVDLVKDDENLTSQEFNKFEDRIYK
A. fulgidus 1          VK---DRPLLNNMIKPDVYSPPDLGAKLAYEVARGGVDIIKDDELLANPEFNRIEERVPK
A. fulgidus 2          VA---DRPIVGTVPKPKVGYSAEEVEKLAYELLSGGMDYIKDDENLTSPAYCRFEERAER
R. rubrum              RPEVDGGLVVGTIIKPKLGLRPKPFAEACHAFWL-GGDFIKNDEPQGNQPFAPLRDTIAL
                           ::.      **.:     ..    .  :      *  *  *:**   .  :    .:


                           SSS  SSSS
Synechococcus PCC6301  VADAIHKSQAETGEIKGHYLNVTAPTCEEMMKRAEFAKELGMP-----IIMHDFLTAGFT 300
M. jannaschii          TLEMRDKAEEETGERKAYMPNITAP-YREMIRRAEIAEDAGSE-----YVMIDVVVCGFS
A. fulgidus 1          FMEAIDRADEEKGEKTLYAVNVTAD-LPEVLENAERAIELGAN-----CLLVNYLATGFP
A. fulgidus 2          IMKVIEKVEAETGEKKSWFANITAD-VREMERRLKLVAELGNP-----HVMVDVVITGWG
R. rubrum              VADAMRRAQDETGEAKLFSANITADDPFEIIARGEYVLETFGENASHVALLVDGYVAGAA
                            .   :  : *.**  .   *:**    *:   . . :         ::  :     *


                                  AA                                A
Synechococcus PCC6301  ANTTLAKWCRDNGVLLHIHRAMHAVIDRQRNHGI-HFRVLAKCLRLSGGDHLHSGTV-VG 360
M. jannaschii          AVQSFRE--EDFKFIIHAHRAMHAAMTRSRDFGI-SMLALAKIYRLLGVDQLHIGTV-VG
A. fulgidus 1          VLRALAE--DESIKVPIMAHMDVAGAYYVSPISGVRSTLILGKLPRLAGADIVVYPAP-YG
A. fulgidus 2          ALEYIRDLAEDYDLAIHGHRAMHAAFTRNAKHGI-SMFVLAKLYRIIGIDQLHIGTAGAG
R. rubrum              AITTARR--RFPDNFLHYHRAGHGAVTSPQSKRGYTAFVHCKMARLQGASGIHTGTMGFG
                       .          .   :  *   ..      .      *   *: *  .:    :    *


                       A               S                          A A
Synechococcus PCC6301  KLEGDKASTLGFVDLMREDHIERDRSRGVFFT--QDWASMPGVLPVASGGIHVWHMPALV 420
M. jannaschii          KMEGGEKEVKAIRDEIVYDKVEAD-NENKFFN--QDWFDIKPVFPVSSGGVHPRLVPKIV
A. fulgidus 1          KAP-----------MMEEKYIEVAKQHRYPFY------HIKPCFPMPSGGIAPIMVPKLV
A. fulgidus 2          KLEG-QK-WDTVQNARIFSEVEYTPDEGDAFHLSQNFHHIKPAMPVSSGGLHPGNLEPVI
R. rubrum              KMEG----ESS--DRAIAYMLTQDEAQGPFYR--QSWGGMKACTPIISGGMNALRMPGFF
                       *               :       .  :         :    *: ***:     :  ..


                           S        AA              S      S       SS
Synechococcus PCC6301  EIFG-DDSVLQFGGGTLGHPWGNAPGATANRVALEACVQARNEGRDLYREGGDILREAGK 480
M. jannaschii          EILG-RDLIIQAGGGVHGHPDGTRAGAKAMRAAIEAIIEGKS------------LEEKAE
A. fulgidus 1          NTLG-KDFVVAAGGGIHAHPDGPAAGARAFRQAIDAAMQGYTD-----------LRKYAE
A. fulgidus 2          DALG-KEIVIQVGGGVLGHPMGAKAGAKAVRQALDAIISAIP-----------LEEHAK
R. rubrum              ENLGNANVILTAGGGAFGHIDGPVAGARSLRQAWQAWRDGVPVLDYAR--EHKELARAFE
                       : :*   : ::   ***  .*  *    .:** : * * :*   ..           *  .  :


Synechococcus PCC6301  WSP--ELAAALDLWKEIKFEFETMDK-- 508
M. jannaschii          EVA--ELKKALEYW--------------
A. fulgidus 1          ENNLQELLKALQL---------------
A. fulgidus 2          QHP--ELQAALEKWGRVTPI--------
R. rubrum              SFPG-DADQIYPGWRKALGVEDTRSALP
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FIG. 2. Deduced amino acid sequence alignment of archaeal (*M. jannaschii* and *A. fulgidus*) and representative form I (*Synecococcus* sp. strain PCC 6301) and form II (*R. rubrum*) RubisCO molecules. Multiple sequence alignments were performed by using ClustalW (29). The accession numbers for each deduced large subunit sequence are as follows: *Synechococcus* PCC 6301, P00880; *M. jannaschii*, Q58632; *A. fulgidus* rbcL1, O28685; *A. fulgidus* rbcL2, O28635; *R. rubrum*, (P04718). Residue identities are marked with an asterisk, conserved substitutions are marked with a colon, and semiconserved substitutions are marked with a period (29). Known active-site residues determined to be within 3.3 Å of the bound transition state analog CABP in the *Synechococcus* PCC 6301 enzyme are labeled A. Where these residues are identical in all three sequences they are in boldface type. Residues known to make contact with small subunits in the *Synechococcus* enzyme are labeled S. The characteristic RubisCO motif sequence, GXDFXKXDE, is shown in boldface and underlined. The alignments were adjusted manually to take into account known structural considerations.

A

B

C
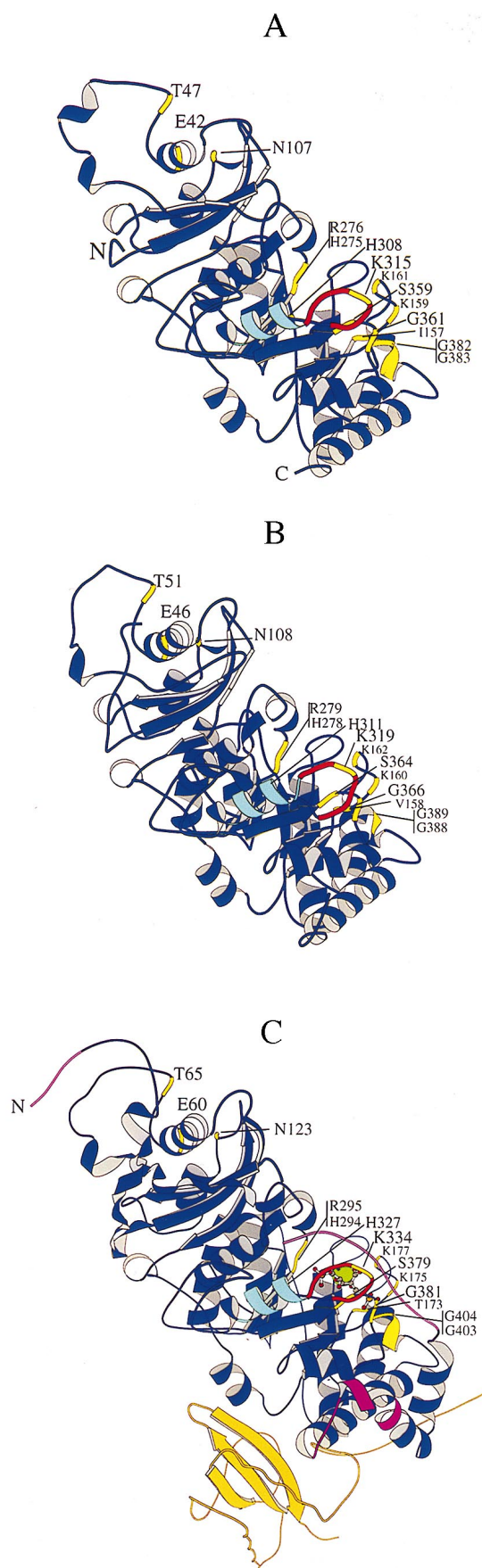
poorly conserved residues or, in fact, do not possess residues that have been previously shown to make contact with small subunits of the form I enzyme (Fig. 2). Where these residues are conserved, most of the conservations are also found in the *R. rubrum* enzyme, which also does not have small subunits (28).

Initially, we focused on the *M. jannaschii* sequence, simply because it was available before those of *A. fulgidus*. It is apparent that the *M. jannaschii* sequence is notably different from those of the form I and II enzymes in the following regions: a large gap in the alignment from position 463 to 474 and a 14-amino-acid truncation at the C terminus relative to the *R. rubrum* enzyme. Similar gaps are found in the *A. fulgidus* deduced sequences. The C-terminal truncation of these putative proteins is of interest since removal of amino acids downstream of Pro-478 of the *R. rubrum* RubisCO yielded an enzyme that lost >99% of its ability to bind CABP and also tended to form an octameric structure (18). In general, the sequences surrounding the 14 active-site residues are more similar between the *Synechococcus* and *M. jannaschii* sequences. However, the region downstream of the catalytically important loop-6 region (residues 364 to 373 of the alignment) is of some interest since this sequence is similar to that of the equivalent region from *R. rubrum* but diverges greatly from those of the *Synechococcus* protein and other form I RubisCOs. Overall, the near absolute conservation of critical residues suggests the strong possibility that selective pressures have maintained the functionality of the *M. jannaschii* protein. Finally, three-dimensional coordinates were obtained for the putative *M. jannaschii* sequence and the *A. fulgidus rbcL2* sequences by homology modeling with the ExPasy server (16). A comparison with the known *Synechococcus* structure clearly demonstrated that the *M. jannaschii* sequence and the *A. fulgidus rbcL2* sequences can be modeled in such a way that all active-site residues found within 3.3 Å of the bound transition state analog CABP (15) are found at the same coordinates in the predicted archaeal RubisCO structures (Fig. 3). Indeed, the active-site residues and many other major features of these structures are virtually superimposable. Major features and differences between the enzymes are highlighted in Fig. 3 (see also the legend to Fig. 2). The confidence level for this model is very high for >90% of the residues.

Properties of recombinant *M. jannaschii* RubisCO. The available "in silico" evidence indicated that the *M. jannaschii* and *A. fulgidus* sequences might encode functional RubisCO. To provide experimental proof for this suggestion, the *M. jannaschii* RubisCO sequence was cloned from total *M. jannaschii* DNA or subcloned from a plasmid previously shown to contain the gene of interest (2). The putative *M. jannaschii* RubisCO

FIG. 3. Tertiary structure prediction of archaeal RubisCO molecules. The predicted tertiary structure of the *M. jannaschii* sequence (A) and the *A. fulgidus rbcL2* sequence (B) is compared to that of the known structure of the *Synechococcus* large subunit (C). The *Synechococcus* small subunit is also shown to the lower left of the structure (in amber). Label sizes and shading reflect the distance from the viewer with the smaller and darker, respectively, being further from the viewer. The main features are highlighted as follows: yellow, active-site residues within 3.3 Å of the bound transition state analog CABP (15) in the *Synechococcus* enzyme and the equivalent residues in the *M. jannaschii* and *A. fulgidus* sequences; red, loop-6 region; cyan, highly divergent α-helix-6 residues; purple, residues that appear to be absent in the *M. jannaschii* and *A. fulgidus* sequences (eight residues at the N terminus of the *Synechococcus* enzyme were not resolved in the structure determination [15] and therefore are not shown here). Mg^{2+} is represented as a green sphere and CABP is represented as a ball-and-stick model in panel C. Images were produced with Molscript (11); no prediction was returned for the first 6 and last 19 amino acids of the *A. fulgidus* structure and the first residue and last 18 amino acids of the *M. jannaschii* structure.
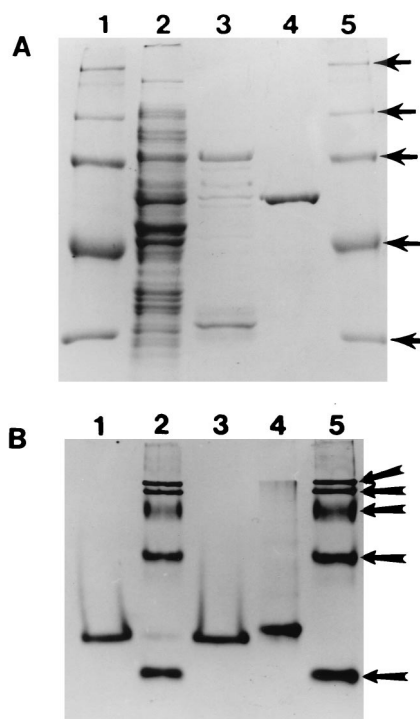
FIG. 4. Purification, subunit structure, and molecular mass of *M. jannaschii* RubisCO. (A) Denatured *M. jannaschii* recombinant RubisCO from crude extracts (lane 2) and the soluble fraction obtained after heating at 85°C (lane 3) and after affinity purification on a nickel-chelate column (lane 4) are shown. Molecular mass markers are shown in lanes 1 and 5 and the arrows correspond (from top to bottom) to proteins of 200, 97, 68, 43, and 29 kDa. (B) Purified *M. jannaschii* protein was electrophoresed on a nondenaturing gel (lanes 1 and 3) and compared to the migration of the *R. rubrum* RubisCO (molecular weight = 110,000 [lane 4]) and several standards (lanes 2 and 5). Arrows (from top to bottom) signify proteins with molecular masses of 669, 440, 232, 140, and 67 kDa.

sequence from the amino terminus had no effect on the enzymatic activity.

This unusual RubisCO was further characterized. In keeping with its novel source, the *M. jannaschii* enzyme was shown to be very stable to high temperatures with no loss of activity at 85°C for up to 60 min; in addition, maximum activity was obtained in the presence of 0.6 M KCl (results not shown). The *M. jannaschii* enzyme produced the expected product, 3-PGA, in the absence of O_2, in a reaction which was coincident with the incorporation of $^{14}CO_2$ into acid-stable product (not shown), yielding a stoichiometry of 1.96 mol of PGA produced per mol of RuBP carboxylated. Carboxylation and 3-PGA formation were specifically inhibited by the RubisCO transition-state analog CABP (Fig. 5A). The production of [^{3}H]3-PGA from a CO_2 fixation reaction mixture containing [1-^{3}H]RuBP was also established (Fig. 5B), further indicating that the *M. jannaschii* enzyme catalyzes a bonafide RubisCO activity.

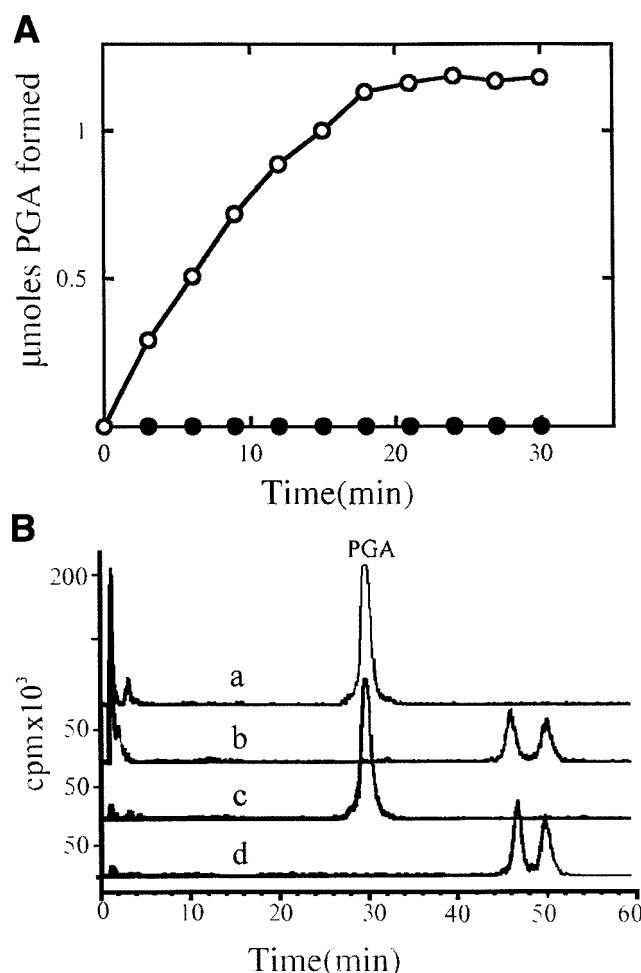Exposure of the enzyme to air under normal laboratory

FIG. 5. (A) Enzymatic determination of product formation (3-PGA) in the absence (○) or presence (●) of the RubisCO transition-state analog CABP (4 μM). (B) Anion exchange chromatographic separation of [^{3}H]3-PGA generated from [1-^{3}H]RuBP after a 1-h reaction at 65°C in the presence (a) or absence (b) of the *M. jannaschii* enzyme or in the presence (c) or absence (d) of RubisCO from *R. rubrum* (27) after 1 h at 25°C. Peaks at the beginnings of the chromatographic profiles represent degraded RuBP produced in this reaction mixture and accelerated by high temperatures (a) and (b), while peaks at the ends of the profiles represent RuBP reduction products produced after the addition of NaBH$_4$ to quench the reaction in the absence of enzyme (b and d).

sequence was then subcloned into vector pProExHTa and then expressed in *E. coli* to produce a (His)$_6$-tagged recombinant protein. Similarly, expression vectors containing the *A. fulgidus* RubisCO sequences have also been prepared (33). The *M. jannaschii* recombinant fusion protein was recovered as a homogeneous preparation from metal-chelate columns (Fig. 4A) and migrated in SDS-polyacrylamide gels as a protein with a molecular weight of about 55,000, consistent with its deduced molecular mass of 51,726 Da. The amino-terminal amino acid sequence of recombinant protein prepared from constructs that do not contain a (His)$_6$-tag sequence (i.e., prepared by using a pK vector) was shown to agree with the amino terminus of the deduced sequence. On a nondenaturing 8% acrylamide gel or a 4 to 20% acrylamide gradient gel (data not shown), the (His)$_6$-tagged protein migrated at a position which would suggest that it is a homodimer with a calculated molecular weight of approximately 105,000 (Fig. 4B). The recombinant *M. jannaschii* protein, whether prepared from a (His)$_6$-tagged or a non-(His)$_6$-tagged vector, possessed specific activities which ranged from 1 to 2 nmol of CO_2 fixed/min/mg of protein in crude extracts. When purified, the fusion protein had a k_{cat} that ranged from 0.5 to 1.6 s^{-1} for several different preparations. This value is somewhat lower than what is usually obtained for RubisCO from eubacteria and eukaryotes (k_{cat} of 3 to 5 s^{-1}) but may reflect our current inability to measure the *M. jannaschii* enzyme under optimum conditions specific for this protein. Specific proteolytic cleavage of the (His)$_6$-tagged
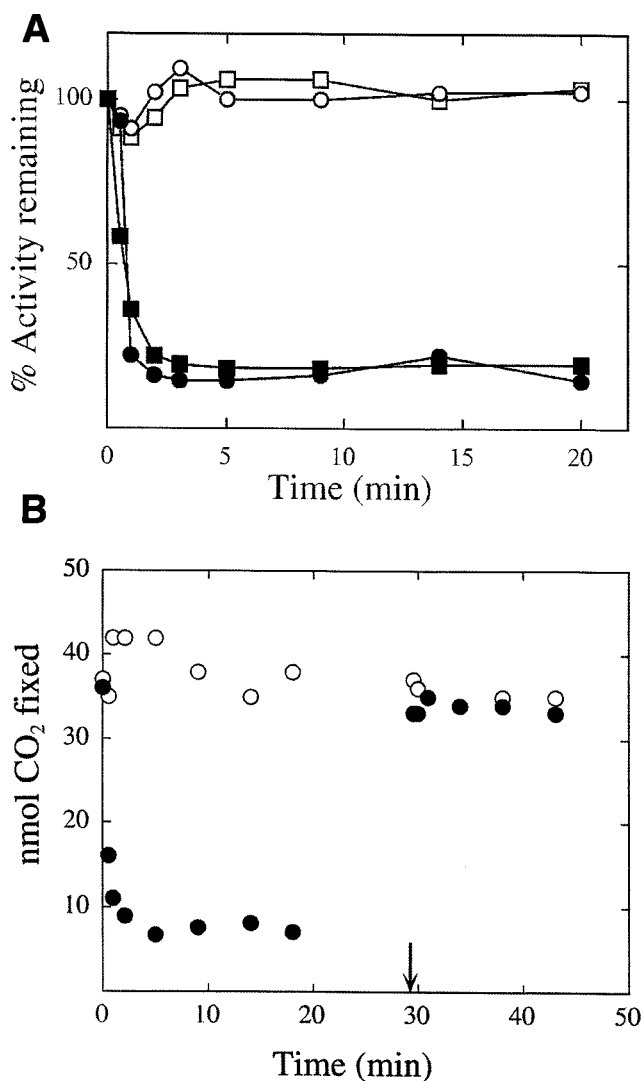
FIG. 6. Effects of molecular oxygen. (A) RubisCO activity after incubation of the *M. jannaschii* enzyme at room temperature for the indicated times in the presence (■, ●) or absence (□, ○) of air (in an argon atmosphere) and assayed anaerobically at 65°C in the presence (●, ○) or absence (■, □) of 10 mM β-mercaptoethanol by a standard 10-min $^{14}CO_2$ incorporation assay. (B) Reversibility of oxygen inhibition for assays run in the absence (○) or presence (●) of air, followed by removal of air and replacement with a nitrogen atmosphere at 18 min and the addition of an O_2 scavenging system containing protocatechuate dioxygenase (7) at 29 min. In all cases, the enzyme was dialyzed in a CO_2-free, O_2-free buffer of 80 mM HEPES (pH 7.2) containing 1 mM EDTA and 10 mM $MgCl_2$.

conditions resulted in considerable loss of enzymatic activity over time (Fig. 6A), precluding routine kinetic measurements in the presence of oxygen. However, inhibition by O_2 was found to be reversible, as removal of this gas followed by the addition of an O_2 scavenging system resulted in the recovery of full enzymatic activity (Fig. 6B). It is also apparent that exposure to air levels of O_2 did not completely inhibit enzyme activity (Fig. 6A). Yet, O_2 binding must be quite efficient since simply diluting air-treated enzyme into an anaerobic assay was not sufficient to reactivate the enzyme (Fig. 6A); activity only recovered after rigorous exchange of the gas and the subsequent addition of an O_2 scavenging system (Fig. 6B).

The observed reversibility of O_2 inhibition, and the known mechanism of RubisCO catalysis (8), suggests that CO_2 and O_2 may be competing for enzyme-bound enediolate, although it is

conceivable that O_2 may interact at a second site on the anoxic *M. jannaschii* enzyme. Certainly, further kinetic studies are very much in order. Most significantly, the low residual activity retained upon exposure of the enzyme to air levels of oxygen suggested that it might be possible to determine if this enzyme catalyzes oxygenase activity, i.e., the oxygenolysis of RuBP. Thus, advantage was taken of known methods to separate and quantitate the specific carboxylase and oxygenase reaction products, which could be determined unambiguously even at low levels of activity by isolating $[^3H]$2-phosphoglycolate (2-PG) and $[^3H]$3-PGA from a reaction mixture containing $[1-^3H]$ RuBP under an air atmosphere. The results of such an experiment indicated that the *M. jannaschii* enzyme catalyzed, albeit weakly and over a long time period, oxygen-dependent formation of 2-PG (Fig. 7). In addition, it would appear that O_2 interacts with the enzyme in such a way that carboxylase activity is diminished, since the level of 3-PGA produced is greatly reduced in these experiments in marked contrast to that of the *R. rubrum* enzyme. Indeed, the levels of 3-PGA and 2-PG produced (Fig. 7), at the concentrations of O_2 and CO_2 employed in this reaction, allow a calculation to be made of the relative CO_2/O_2 substrate specificity (τ) of the archaeal enzyme (7). For three separate experiments, with assays performed in the presence of air or molecular oxygen, the *M. jannaschii* enzyme yielded a CO_2/O_2 specificity factor of about 0.5, the lowest value ever reported for RubisCO from any source. The calculated specificity value of the *M. jannaschii* enzyme, and the long time required to produce the reaction products at room temperature in the presence of oxygen, resembles the low specificity and low activity obtained for a mutant *R. rubrum* enzyme (13). It should be noted that assays were performed at room temperature, far from the optimum temperature for activity (65°C) of the *M. jannaschii* enzyme. This was done to minimize the degradation of RuBP, which was exacerbated at this temperature in the high-salt environment of the specificity
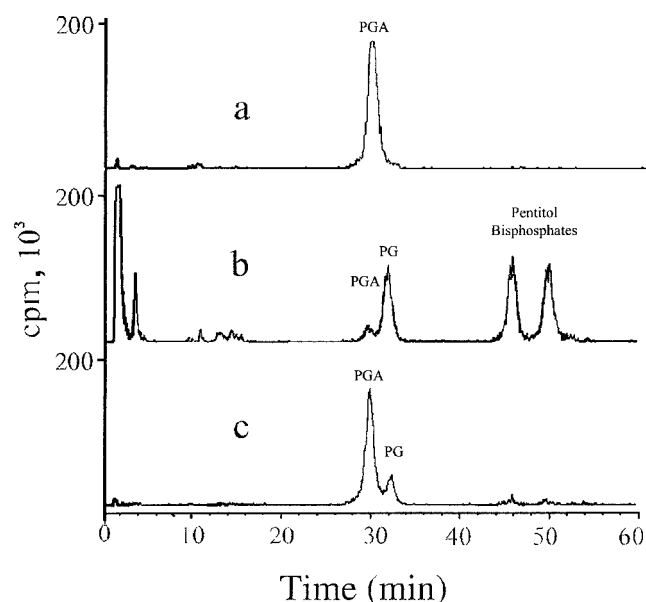

FIG. 7. Anion exchange chromatographic separation of products from a reaction containing $[1-^3H]$RuBP and the recombinant *M. jannaschii* enzyme incubated in the absence of air (a) or presence of air (b) at room temperature. In profiles a and b, the reaction was allowed to continue for 5 days. A similar reaction containing the *R. rubrum* RubisCO was performed in the presence of air for 60 min at room temperature (c). Assays were run at room temperature to limit the time-dependent degradation of RuBP that occurs under these conditions.

assay. Further rigorous CO_2/O_2 specificity and kinetic studies of this archaeal enzyme are obviously in order and should provide answers as to why this enzyme has such low substrate specificity and low activity in the presence of oxygen.

In conclusion, analysis of sequences which encode putative RubisCO proteins in *M. jannaschii* and *A. fulgidus* indicate, especially for *M. jannaschii*, the potential for the formation of a catalytically active enzyme. The preparation of homogeneous *M. jannaschii* recombinant protein with the capacity to catalyze a demonstrable RubisCO reaction, at a k_{cat} that is somewhat lower, yet approximates, that obtained for previously studied proteins, indicates that this recombinant enzyme is indeed functional. These analyses, however, do not predict what physiological role RubisCO might have in *M. jannaschii* or *A. fulgidus*. Genomic sequences obtained from these organisms do not show the presence of a recognizable gene(s) for phosphoribulokinase (21), the enzyme needed to complete the CBB pathway and also to generate the CO_2 acceptor RuBP. In part, this might be because only few phosphoribulokinase sequences are available in the current database; those that are available from eubacteria and eukaryotes show as little as 13% identity (25). Thus, phosphoribulokinase sequences that might yield a potential match to one or more open reading frames of unknown function in the genomes of *M. jannaschii* and *A. fulgidus* (2, 10) may be unrecognizable. In any case, the *M. jannaschii* enzyme purified here represents a form of RubisCO that has hitherto not been encountered and may be limited to anoxic extremophile representatives of the archaea. Indeed, determining how this enzyme functions at high temperatures and copes with O_2, a substrate that the *M. jannaschii* or *A. fulgidus* enzymes should never encounter, has considerable fundamental interest and may lead to an understanding of how "conventional" RubisCO molecules discriminate between CO_2 and O_2. In this regard, the apparent unusually high sensitivity of the *M. jannaschii* enzyme to O_2, a molecule that serves as both substrate and inhibitor, is unique to this source of RubisCO. As the signature property of RubisCO, CO_2/O_2 specificity plays an important role in global productivity and CO_2 sequestration and is a property that seems to have evolved to different extents for different sources of RubisCO (9, 20). Further studies of the archaeal enzyme are likely to be particularly cogent and should provide answers as to how the active site of this important enzyme has adapted to function at various levels of CO_2 and O_2 in different organisms in diverse environments.

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